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(54) Title: CELL CULTURE MEDIUM

(57) Abstract

A basal serum-free medium and a hematopoietic cell growth and differentiation-promoting serum-free medium based thereon are provided for the maintenance, cultivation, growth and differentiation of erythroid progenitor cells, other hematopoietic progenitor cells, and leukemia cells in which the effects of various growth factor compounds can be quantitatively evaluated. Both media are wholly serum-free and contain no intrinsic growth factor compounds. The hematopoietic growth and differentiation medium consists essentially of the basal serum-free medium to which has been added at least one primarily but not exclusively growth promoting agent selected from heme or hemin, interleukin-3 and recombinant human stem cell factor (and optimally all of them), and at least one primarily but not exclusively cell differentiation promoting agent selected from erythropoietin (Epo), insulin-like growth factor (IGF) and a retinoid (and optimally including all of them).

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CELL CULTURE MEDIUM

This invention relates to culture medium and more particularly to a culture medium for the maintenance and growth of erythroid progenitor cells, other hematopoietic progenitor cells and leukemia cells.

Mammalian erythropoiesis is normally regulated in vivo by Epo (erythropoietin), a glycoprotein hormone responsible for the differentiation of red blood cells and for the viability and proliferation of their progenitors. Years after the introduction of culture media for the growth of the erythroid progenitors CFU-E (colony forming unit-erythroid) and BFU-E (burst forming unit-erythroid) it was found that fetal bovine serum (FBS) contained signifi- cant quantities of an Epo-like activity.[1] Originally, this was believed to be due to the presence of Epo in FBS, but radioimmunoassays of Epo in FBS found only minute amounts of the hormone.[2,3] Subsequently, it was shown that the Epo- like activity of FBS was abrogated by an antiserum directed against insulin-like growth factor I(IGF-I), but not by anti-Epo antiserum.[3] The same investigators also found that purified human IGF-I could stimulate colony-formation by CFU-E from murine fetal liver and adult bone marrow cells in a "serum free" "SF" medium.[4]   Further studies showed that IGF-I enhanced both CFU-E- and BFU-E-derived colony formation from human bone marrow and peripheral blood in "SF" medium as well[5].

To determine the cellular and molecular mechan- isms that regulate erythropoiesis, there is needed a truly SF culture system for circulating erythroid progenitor cells which would be defined with respect to all activities that are stimulatory for erythropoiesis. Simple depletion of serum from a culture medium does not necessarily remove from it all undefined serum factors. Major vehicles for

the uncontrolled presence of such factors in culture media, in the absence of added serum or plasma, are the albumin preparations commonly employed, most typically Cohn's Fraction V. These have been shown to provide a mitogenic factor(s)[6,7] referred to as BPA ("Burst Promoting Activity"[8]) which has so far remained molecularly unidentified. In fact, most so-called "SF" media in the current literature use bovine serum albumin (BSA) preparations that are known to be contaminated with such activities. Effectively, serum residues provide a "dirty background" which precludes accurate and reliable study of the precise effects of added growth factors. Accordingly, in this specification, the term "serum-free" or "SF" is used in quotation marks in instances where reference is made to a medium reported originally to be serum-free but subsequently discovered to be still serum-contaminated.

In our laboratory, we have previously shown that treatment of BSA with activated charcoal removes its BPA-like activity.[6] Using a fatty-acid-free and globulin-free BSA (FAF, GF BSA) preparation in a SF medium for the growth of BFU-E from bone marrow, Ogawa and co-workers[9] demonstrated that under these stringent conditions, a defined exogenous source of BPA-like activity, interleukin-3 (IL-3) or GM-CSF, had to be added in order to obtain growth of day-14 erythroid bursts. With this SF medium, however, we were unable to grow erythroid bursts or colonies from circulating progenitor cells.

The study of erythropoiesis, including the study of the growth and proliferation of leukemia cells, so important to the development of understanding and eventual control of this disease, requires the provision of a cell growth medium which ensures optimum growth of the cells under study. It also requires the provision of a cell growth medium free from unknown ingredients which might

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affect the cell growth characteristics. Only with such a
growth medium can the researcher satisfactorily study the
effects of other growth-promoting or -inhibiting factors on
cell proliferation and differentiation, as well as study
the precise effects of toxic molecules.

It is an object of the present invention to
provide a novel cell culture medium for hemopoietic and
leukemia cells.

The present invention, from a first aspect, is
based upon the provision of a basal serum-free medium
(BSFM) for erythroid and other hematopoietic progenitor
cells namely a medium in which the cells will not prolifer-
ate. By the identification and provision of such a basal
serum-free medium, there is provided to the user a medium
to which other ingredients may be added for the specific
purpose of determining whether or not such ingredients
constitute growth and/or differentiation factors for the
cells under study. From a second aspect, the present
invention provides a growth and differentiation medium
which can be optimised for such cells, the medium being
constituted by the basal serum-free medium with certain
well-defined additives designed to ensure optimum growth
and differentiation of the cells therein. With this
optimum growth and differentiation medium, the user can
test the effects of various compounds on the cell growth
and differentiation characteristics of hematopoietic and
leukemia cells. The concept of distinguishing between a
basal serum-free medium (BSFM) and a cell growth and
differentiation medium provides the basis of the present
invention.

Thus, according to the first aspect of the
invention, there is provided a basal serum-free medium for

erythroid and other hematopoietic progenitor cells and leukemia cells, consisting essentially of:

a minimum essential medium;

an effective amount of each of the four deoxyribonucleosides adenine deoxyriboside, thymine deoxyriboside, guanine deoxyriboside and cytosine deoxyriboside;

an effective amount of each of the four ribonucleosides adenine riboside, uridine riboside, guanine riboside and cytosine riboside;

an effective amount of L-glutamine;

an effective amount of deionized, fatty-acid free and globulin-free bovine or human serum albumin or recombinant human albumin;

an effective amount of a human or bovine transferrin;

an effective amount of a phosphatidyl choline;

an effective amount of a C_{16} - C_{24} unsaturated fatty acid;

an effective amount of a cholesterol;

an effective amount of d-α-tocopherol or an ester thereof;

an effective amount of at least one bio-acceptable antioxidant;

and an effective amount of at least one antibiotic substance effective to protect the cells against stray infections.

According to the second aspect of the present invention, there is provided a serum-free culture medium for the growth and differentiation of erythroid and other hematopoietic progenitor cells and leukemia cells, said medium being free from indigenous cell growth factors but adapted to receive extraneous cell growth influencing materials for determination of the effects thereof on erythroid and other hematopoietic cells and leukemia cells

and for attaining optimal growth thereof, said culture medium consisting essentially of:

> - a basal serum-free medium as defined above;
> - an effective amount of at least one growth promoting agent selected from the group consisting of heme or hemin, interleukin-3, and recombinant human stem cell factor;
> - an effective amount of at least one cell differentiation-promoting agent selected from the group consisting of erythropoietin, insulin-like growth factor, and a retinoid.

It will be understood that the agents referred to as growth-promoting agents may in addition have some cell differentiation-promoting activity, and that the agents referred to as differentiation-promoting agents may also have some growth-promoting activity.

In this novel and improved SF growth and differentiation medium which we have developed, the concentration of each component has been optimized. With it, we have been able to produce erythroid bursts from the mononuclear cells of human peripheral blood with higher efficiencies than those of either the serum-containing or the "serum-free" media reported in the literature. This medium, which uses BPA-free FAF, GF BSA, and defined sources of BPA-like activity, has allowed us to study the effects of various growth factors such as IGF-I upon circulating erythroid progenitors grown in vitro. We have found that IGF-I does not behave as a BPA, inasmuch as it cannot replace IL-3 in the presence of Epo. The effect of IGF-I in this medium was found to be interchangeable with that of Epo, though at higher concentration, thus revealing an Epo-independent pathway for erythropoiesis in vitro by circulating progenitors of the normal human adult. This mechanism, if operat-

ive in vivo, may become important under conditions in which endogenous Epo levels are low.

The BSFM provides no complications such as intrinsic presence of substances, in unknown or even trace quantities, which might have cell growth effects, inhibitory or stimulatory, on hematopoietic progenitor cells or leukemia cells. The medium accordingly provides an ideal test medium for evaluation, optimization and development of growth and differentiation factors that act on hematopoietic progenitor and leukemia cells, as well as elucidation of the mechanism whereby they operate.

According to another aspect, the present invention provides a process of determining the effect of a test substance on the growth characteristics of erythroid and other hematopoietic progenitor cells or leukemia cells, which comprises culturing said cells under predetermined, controlled conditions in a serum-free growth and differentiation medium as defined above and to which has been added a predetermined quantity of said test substance, and recording growth characteristics of said cells in the culture medium.

The SF growth and differentiation medium as defined above provides for the optimal growth in vitro of normal human peripheral blood progenitor cells (BFU-E and CFU-E), normal human bone marrow progenitor cells (BFU-E and CFU-E), normal human bone marrow multipotential hematopoietic stem cells (CFU-GEMM), normal human bone marrow granulocyte/macrophage progenitor cells (CFU-GM), peripheral blood erythroid progenitor cells (BFU-E and CFU-E) from patients with polycythemia vera, bone marrow granulocyte/macrophage progenitor cells (CFU-GM) and erythroid progenitor cells (BFU-E) from patients with Diamond-Blackfan anemia, and human polycythemia vera, and

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B- and T- leukemia cell lines. The growth of certain leukemia cell lines may not require all components of the complete growth and differentiation medium defined above.

FIGURE 1 is a bar graph presentation of the results of Example 1 below;

FIGURE 2 is a bar graph presentation of the results of Example 2 below, showing variation in the number of erythroid bursts and their component colonies derived from normal human PB MNC with different combinations of hemin and retinyl acetate (in the presence of rHu IL-3, and rHu Epo and rHu IGF-I) in an improved SF medium containing a "clean" BSA and in a serum-containing medium with "dirty" BSA;

FIGURE 3 is a bar graph presentation of the further results of Example 2 below, and illustrating the effect of ATRA on early erythroid colony formation by circulating progenitors in an improved SF medium;

FIGURE 4 is a graphical presentation of further results obtained in Example 3 below, namely the relation between number of erythroid bursts produced and number of PB MNC plated in an improved SF medium containing 5.5 ng/ml IL-3, $3 \times 10^{-8}M$ retinyl acetate, $3 \times 10^{-8}M$ rHu IGF-I, 3.0 U/ml Epo and 0.1 mM hemin;

FIGURE 5 is a graphical presentation of the results obtained from Example 4 below. It shows the number of burst-component colonies formed from normal PB MNC in an improved SF medium as a function of rHu IL-3 concentration. Culture medium contained 3.0 U/ml rHu Epo, $3 \times 10^{-8}M$ retinyl acetate, $3 \times 10^{-8}M$ rHu IGF-I and 0.1 mM hemin

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FIGURE 6 is a graphical presentation of further results obtained from Example 4 below, namely the variation in the number of bursts (•) and their component colonies (O) derived from PB MNC as a function of hemin concentration in an improved SF medium containing 5.5 ng/ml rHu IL-3, 3.0 U/ml Epo, $3 \times 10^{-9}M$ IGF-I and $3 \times 10^{-6}M$ retinyl acetate. Each colony had at least 50 hemoglobinized cells;

FIGURE 7 is a graphical presentation of the results obtained from Example 5 below, and shows the number of burst-component colonies derived from PB MNC in an improved SF medium as a function of rHu IGF-I concentration with (O) and without (•) retinyl acetate ($3 \times 10^{-6}M$). The medium contained 5.5 ng/ml rHu IL-3, 3.0 U/ml rHu Epo and 0.1 mM hemin;

FIGURES 8A, 8B, and 8C are graphical presentations of the results obtained from Example 6 below;

FIGURES 9A and 9B are graphical presentations of additional results obtained from Example 6 below, namely comparisons of numbers of erythroid bursts derived from PB MNC as a function of rHu IGF-I or rHu Epo concentration in an improved SF medium, and comparison of normalized values from the above data. Conditions are identical to those of Fig. 8;

FIGURE 10 is a graphical presentation of additional results from Example 6 below, namely the effect of the addition of polyclonal anti-Epo antibody HCC-1400 to an improved SF medium with different combinations of rHu Epo and rHu IGF-I. The HCC-1400 antibody was used at 1:50 final dilution; at this concentration it is known to neutralize 6 U/ml of Epo. Molar concentrations of Epo and IGF-I employed were respectively 1.8 nM (6 U/ml) and 300 nM;

FIGURE 11 is a graphical presentation of the results obtained according to Example 8 below.

FIGURES 12A 12B, 12C and 12D are graphical representations of the results of Example 9 below.

FIGURE 13 is a graphical presentation of results obtained according to Example 10 below.

FIGURE 14 is a graphical presentation of further results obtained according to Example 10 below.

FIGURE 15 is a graphical presentation of results obtained according to Example 11 below.

FIGURE 16 is a graphical presentation of further results obtained according to Example 11 below.

FIGURES 17A and 17B are a graphical presentation of the results obtained according to Example 12 below.

The basal serum-free medium and the SF growth and differentiation medium as defined above with their minimum number of components provide media in which useful results on cell viability, growth and differentiation, capable of meaningful scientific interpretation can be obtained. According to the preferred embodiments of the invention, however, additional ingredients are added as discussed herein, to produce an optimal SF medium.

The present invention provides a novel improved SF medium for the growth and differentiation of human erythroid progenitor cells from peripheral blood. The activity in this medium of each of its major components has been systematically investigated. It has been found that in the presence of a "clean", fatty acid-free and globulin-

free crystallized albumin, rHu IL-3 and rHu Epo were barely sufficient to support the production of erythroid bursts; the bursts were small, developed in minimal numbers, and required benzidine staining for their confirmation. Full maturation of the erythroid bursts necessitated the addition of hemin, which made possible the direct scoring of hemoglobinized bursts in situ. Optimal growth was attained when IGF-I and retinyl acetate were added. It is believed that this is the first SF medium which, using a BPA-free BSA and a defined source of BPA, fully supports the production of bursts in vitro by circulating erythroid progenitor cells from the normal human adult.

It is very important to make the erythroid culture medium completely free of serum products inasmuch as these introduce not only undefined activities that promote growth, but also undefined inhibitory factors, as our results obtained with a "dirty" albumin and serum illustrate in the following examples. A medium containing FBS and Cohn's Fr. V BSA, even after it had been improved by the addition of hemin, retinyl acetate and IGF-1, had a 33 - 72% lower day-14 colony-forming efficiency than our own improved SF medium. This is demonstrated in Example 2 below, and illustrated in accompanying Figure 2.

As described above, the first aspect of the invention described and claimed herein comprises basal serum-free medium in which hematopoietic cells will not proliferate. The first component thereof is minimum essential medium, a term well understood by those skilled in the art of cell culturing. Specific preferred examples thereof include α medium, IMDM, and Iscove's modified Dulbecco's medium. Such minimum essential medium is then modified according to the invention to render it specifically suitable for the maintenance of hematopoietic cells and leukemia cells. For this purpose, there are added to it

the four deoxyribonucleosides, the four ribonucleosides and the other additives referred to above. The phosphatidyl choline which is used is suitably L-α-phosphatidyl choline dipalmitoyl synthetic. The C_{16} -C_{24} unsaturated fatty acid is suitably linoleic acid or oleic acid. The cholesterol component is suitably porcine liver cholesterol. The antioxidant is suitably beta-mercaptoeth-anol or α-thioglycerol, or most preferably a mixture of both. One or more antibiotic substances are also included, to protect the medium against infection by airborne or other stray bacteria. Suitable such antibiotics include sodium penicillin G and streptomycin sulphate. Preferably a combination of these two antibiotics is used. The semi-solid matrix material is suitably a carbohydrate such as methyl-cellulose, agar or agarose, with methyl-cellulose being most preferred.

Suitable relative amounts of the various constituents of the basal serum-free medium are, in final molarity or in grams per milliliter of final volume:

each deoxyribonucleoside: from 1 μg/ml to 100 mg/ml, and preferably from 5 to 10 mg/mL;

each ribonucleoside: from 1 to 100 μg/mL, and preferably from 5 to 10 mg/mL;

L-glutamine: from 0.1mM to 20 mM and preferably from 1 to 2 mM;

albumin: from 1 mg/mL to 100 mg/mL and preferably from 10 to 30 mg/mL;

transferrin: from 1 μg/mL to 1 mg/mL and preferably from 27 to 270 μg/mL;

phosphatidyl choline: from 0.1 μg/mL to 100 μ/mL and preferably from 5 to 10 μg/mL;

fatty acid: from 0.1 to 100 μg/ml and preferably from 2 to 10 μg/mL;

cholesterol: from 0.1 to 100 µg/mL and preferably from 1 to 10 µg/mL;

antioxidant: from 1 µM to 1mM and preferably from 0.1 to 0.2 mM;

antibiotic: from 0.1 µg/mL to 250 µg/mL and preferably from 25 to 100 µg/mL;

matrix material: from 0.1 mg/mL to 10 mg/mL and preferably from 1 to 3 mg/mL.

In making up the medium for optimal cell growth and differentiation, as determined by the maximum yield of colonies and number of cells per colony over a given period of time, from a given number of cells plated out, in an easily studiable condition, together with a maximized degree of hemoglobinization by the cells, the additional ingredients should be added to the basal serum-free medium, in the following approximate amounts:

hemin or heme: from 50 µM to 1 mM and preferably from 200 µM to 500 µM;

retinoid: from 0.1 nM to 0.1 nM and preferably from 1 nM to 100 nM;

stem cell factor: from 0.5 ng/mL to 1 mg/mL and preferably from 25 to 100 ng/mL;

interleukin-3: from 0.1 ng/mL to 1 mg/mL and preferably from 5 to 25 ng/mL;

erythropoietin: from 0.1 ng/mL to 1 mg/mL and preferably from 10 to 30 ng/mL;

insulin-like growth factor: from 1 pg/mL to 1 mg/mL and preferably from 20 to 100 ng/mL.

EFFECTS OF INDIVIDUAL COMPONENTS OF THE GROWTH MEDIUM BPA: IL-3, HEMIN AND SCF

The problem of providing a defined BPA required for the growth of erythroid bursts has been a major stumbl-

ing block in the development of truly SF media. Most
recipes are in fact merely serum-deprived media,[5, 10-13] as
they call for the use of Cohn's Fr. V BSA, which is known
to be contaminated with BPA, as well as lipids, small
proteins and other low molecular weight molecules.[13]
Delipidation with activated charcoal not only removes
lipids but other small molecules as well, and has resulted
in the loss of the erythropoietic activity associated with
albumin.[6,7] A source of BPA then had to be provided, either
by the addition of leukocyte conditioned media[7] or serum
lipoproteins of low to intermediate density.[14]

Investigators have reported BPA-like effects in
their media, even though the albumin had been treated with
activated charcoal; the source of such BPA was traced to
the semi-purified Epo preparations utilized[10] and, when rHu
Epo was used, to the accessory cells of bone marrow
(monocytes, T-cells, fibroblasts)[7]. It has been reported
that accessory cells of bone marrow produce BPA.[7, 15-17] When
we did not remove accessory cells from our PB MNC prepara-
tions, as described in Example 1 below, these cells did not
appear to provide significant amounts of BPA in our system,
judging from the fact that in the absence of added IL-3 and
hemin, no bursts developed (Fig. 1).

However, production of erythroid bursts by
progenitors among PB MNC was completely abolished by
removal of adherent cells in serum-free medium containing
IL-3 and hemin but no SCF; addition of rHu SCF partially
restored burst formation. Thus either non-adherent
erythroid progenitor cells have a requirement for another
growth factor(s) normally secreted by adherent cells, or
else intimate cell-cell contact interactions between
adherent cells and erythroid progenitors are required for
burst production.

In our novel culture system we have confirmed the well-documented BPA-like activity of IL-3 for human erythroid growth _in vitro_; its introduction into our medium as a defined BPA made up for the loss of an undefined BPA resulting from our use of a "clean" BSA preparation and recombinant EPO. In the absence of SCF, the BPA in our SF culture medium appears in fact to be supplied by two defined entities, rHu IL-3 and purified hemin as demonstrated in Example 1 and Figure 1, and Example 2 and Figure 2, reported below.

Hemin (iron protoporphyrin IX) is a relatively small (652 dalton) molecule, known to act in the process of normal erythropoiesis. In addition it has distinct proliferative effects in hematopoietic culture. Hemin has been shown to enhance the proliferation of both the multipotential stem cells CFU-GEMM and the early erythroid progenitor cells BFU-E in serum-containing or serum-deprived (i.e. containing undefined BPA's), "SF" cultures of murine and human bone marrow.

Recently, a novel growth factor termed stem cell factor, SCF, has been reported to stimulate primitive pluripotential hemopoietic progenitors in mice[18,19], rats[20] and humans[18-21]. Several groups have isolated SCF, also termed mast cell growth cell factor (MGF) and have shown it to be the ligand for the c-kit receptor protein. The product of the c-kit proto-oncogene has tyrosine kinase activity and maps to the mouse W locus on chromosome 5. These findings demonstrated that the genetic anemias of W/W[v] and Sl/Sl[d] mice were interconnected, with the former having defects in the c-kit receptor and the latter defective production of SCF, the c-kit receptor ligand being the product of the Steel (Sl) locus. Besides the soluble forms of SCF, membrane-bound forms have also been reported[22]. Serum-containing (SC) studies of the effects of SCF on

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highly enriched, adherent cell-deprived, human bone marrow (BM) BFU-E preparations have shown that it can potentiate primitive hemopoietic colony-formation, and have suggested that it synergises with diverse cytokines, among which are Epo and IL-3[20-21]. No comparable studies exist for human BFU-E from peripheral blood.

The novel serum-free (SF) growth and differentiation medium of the present invention is capable of supporting the growth of circulating erythroid progenitors. However, even though the medium is capable of supporting burst formation at low cell density, removal of adherent cells from the peripheral blood mononuclear cell (PB MNC) suspensions prevents burst formation in SF medium. In accordance with another feature of the present invention, it has been found that recombinant human stem cell growth factor (SCF) can replace the stimulatory activity of adherent cells and thus at least partially restore burst-formation. This is further described in Example 9 below.

IGF-1 AND EPO

The present invention provides strong indications that the effect of IGF-I on burst formation is not limited simply to potentiation of the effect of any Epo that might still be present in the medium. It has been shown that when defined BPAs (IL-3 and hemin) are provided, the basal serum-free medium according to the invention is entirely free of any Epo-like activity capable of inducing erythroid differentiation. Moreover the present work shows that in the presence of an antibody to Epo, IGF-I is fully capable of supporting erythropoietic burst production.

Since experiments according to the present invention were unable to obtain growth of BFU-E with a combination of Epo and IGF-I in the absence of IL-3, hemin,

and retinyl acetate, it appears that IGF-I does not have a
BPA-like effect upon early BFU-E. This fits with the known
incapacity of IGF-I to act as a competence factor, i.e. it
cannot recruit quiescent cells into the cell cycle. We
have also been able to confirm in our culture system that
rHu Epo has no BPA-like activity either.*

Thus IGF-I can function in vitro on its own as an
erythroid differentiation factor for BFU-E from normal
peripheral blood. IGF-I must also function as a growth
factor for the progenitors of burst-component colonies. We
have found that the cellularity of these colonies was
greatly increased by IGF-I whether or not Epo or retinyl
acetate were present. This indicates that IGF-I can
stimulate cell proliferation within the developing burst,
either by promoting self-renewal of the early BFU-E itself,
or by targeting progenitor cells that are later in the
differentiation sequence than the early BFU-E and promoting
their proliferation. The work in support of the present
invention appears to represent the first demonstration of
Epo-like activity of rHu IGF-I on burst and colony forma-
tion from human PB MNC in a demonstrably SF medium.

The SF medium of the present invention provides
the opportunity to investigate the sensitivities of human
circulating erythroid progenitors to the growth factors
that are active in erythropoiesis in vitro. We found that
these progenitors required a 100-fold higher concentration
of IGF-I than of Epo to reach maximal stimulation (Figs. 8,
9). We further found that the result of the combined
addition of Epo and IGF-I was only partially additive (Fig.
1). The combination of Epo and IGF-I in the presence of
IL-3 and hemin gave a greater day-14 colony-forming effi-
ciency than either factor alone, but less than the sum of
their separate effects (Fig.1). This observation suggests
that there may be two classes of BFU-E-derived erythroid

progenitors which overlap with respect to their sensitiv-
ities to each of these factors.

The observation that IGF-I (in the presence of
IL-3) can completely replace Epo constitutes strong evi-
dence for the existence of an IGF-I-dependent mechanism for
proliferation and differentiation of normal circulating
BFU-E, which can operate in vitro when Epo levels are low
or absent. That such an Epo-independent mechanism might
also function in vivo is suggested by the finding that
plasma from a patient with chronic renal failure and low
Epo levels could nevertheless support erythropoiesis
because of its IGF-I content.[23]

VITAMIN A

We have found that the addition of Vitamin A
(retinyl) acetate or all-trans retinoic acid at physiologi-
cal concentration[24,25] (in the absence of Epo) greatly
enhances the effect of IGF-I on erythropoiesis in vitro.
Even at ineffective concentrations of IGF-I added to the
medium, this vitamin is responsible for the expression of
a background number of day-14 erythroid colonies, and it
synergizes with hemin in stimulating production of
increased numbers of these colonies. Retinyl acetate or
all-trans retinoic acid thus appears to act as a potentia-
tor of the functions of other growth factors in the SF
medium.

The invention is further described for illustra-
tive purposes in the following specific examples, consti-
tuting the "Most Preferred Embodiments".

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MATERIALS AND METHODS

CELL PREPARATIONS

After informed consent, peripheral blood was obtained by venipuncture from healthy donors and was immediately placed in α-minimal essential medium (α-MEM) containing 2% fetal bovine serum (FBS) (#SP80219, Gibco, Grand Island, N.Y.) and 10 U/ml of preservative-free sodium heparin (#820 5077 MF, Gibco). Peripheral blood mono-nuclear cells (PB MNC) were separated by Ficoll-Hypaque (Pharmacia, Montreal, P.Q.) density-gradient centrifugation at 400 xg for 40 min.

Adherent cells were removed by 90 min exposure to the plastic of 50 ml Falcon Tissue Culture Flasks (#3013, Becton Dickinson, Rutherford, New Jersey) in the presence of 2% FBS + α-MEM, without agitating the flask at the time of removal of the cell suspension. Cell suspensions were washed three times (400xg, 10 min), the first wash in the presence of 2% FBS + α-MEM, and the subsequent two washes in α-MEM alone. Cell counts were made with the Trypan Blue (0.4%, #630-5250, Gibco) dye-exclusion method.

CLONAL CELL CULTURE

BASAL SERUM-FREE MEDIUM

Serum-free culture of PB MNC was performed with a modification of the technique previously described,[6] in flat bottomed 1.5 x 1.0 cm plastic wells (#76-000-04, Flow Laboratories, MacLean, VA, now discontinued; Nunclon Delta, Nunc, Roskilde, Denmark, S1-24 well multidishes - #1-43982 can also be used). Between 5 x 10⁴ and 1 x 10⁵ PB MNC were plated in 0.5 ml of final culture medium containing α-MEM, 0.8% of 1,500 centipoise methylcellulose (Methocel A4M, premium grade, Dow Chemical Co., Midland, MI), 1% fatty

acid- and globulin-free crystallized BSA (Sigma) which was subsequently deionized with analytical grade Ion Exchange Resin (AG 501-X8(D), BioRad Labs, Richmond, CA), 2×10^{-4}M β-mercaptoethanol (BDH Biochemicals, Poole, England), 270 μg/ml fully iron-saturated bovine transferrin (Sigma), 7×10^{-7}M d-α-tocopherol (Clinic Products, Windsor, Ont.), 8 μg/ml L-α-phosphatidyl choline dipalmitoyl synthetic (Sigma), 5.6 μg/ml oleic acid (Sigma), 7.8 μg/ml porcine liver cholesterol, grade 1 (Sigma), 10 μg/ml of each of the four deoxy- and ribonucleosides (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml penicillin G and 50 μg/ml streptomycin sulfate (Gibco) (this combination of ingredients constituting a specific example of a basal serum-free medium according to the invention) and 3×10^{-8}M retinyl acetate (Nutritional Biochemicals Corp., Cleveland OH).

RECOMBINANT AND OTHER GROWTH AND DIFFERENTIATION FACTORS

E. coli-derived recombinant human somatomedin-C (referred to as rHu IGF-I) having the natural amino acid sequence was purchased from Amersham, Oakville, Ont., or from AMGen, Thousand Oaks, Calif. The recombinant human preparations of erythropoietin (rHu Epo) and Interleukin-3 (rHu IL-3) were from AMGen. Bovine type 1 hemin (ferric chloride protoporphyrin IX) was purchased from Sigma (#H-2250), with ~ 97% purity by spectrophotometric assay. Retinyl acetate was from Nutritional Biochemicals Corp., Cleveland, OH. Recombinant human SCF was a gift from Dr. A. Bernstein (S. Linenfeld Research Inst., Ont.). The final concentrations of these growth and differentiation factors used in the optimal medium were as follow: rHu IGF-I 0.26μg/ml to 2.6 μg/mL (3×10^{08} to 3×10^{-7}M), rHu Epo 3.0 U/ml (27 ng/mL, 9×10^{-10}M), rHu IL-3 5.5 ng/mL (2×10^{-10}M), rHu SCF 100 ng/mL (3nM), retinyl acetate or all-$\underline{trans}$ retinoic acid 3×10^{-8}M, and hemin 65.2 or 163.0 μg/mL (1.0 or 2.5×10^{-4}M).

Petri dishes containing the wells were incubated at 37°C in a humidified atmosphere and 5% CO_2 for 7 to 9 days for erythroid colonies and 14 to 16 days for erythroid bursts. All erythroid colonies and bursts were scored by *in situ* observation with an inverted microscope.

ANTI-EPO ANTIBODY

Polyclonal rabbit anti-Epo antibody HCC-1400 was obtained from Terry Fox Laboratory, Vancouver, B.C., and used at a final dilution of 1:50, at which concentration it neutralizes 6 U/ml of human Epo.

CRITERIA FOR SCORING OF ERYTHROID COLONIES AND BURSTS

An erythroid burst is defined as either a single colony or a cluster of "burst-component" or "day-14" colonies, each having at least 50 hemoglobinized cells, scored at days 14 to 16 of growth. Hemoglobinized colonies with <50 cells and separated from one another could also be observed; even when they appeared to belong to a single burst, their counts were not included in the counts of bursts. Contiguous colonies which collectively comprised at least 50 cells were scored as a burst. Burst-component colonies, also referred to as "day-14 colonies" or "subcolonies", were easy to count and their numbers could be considered reliable even when crowding of the cultures made the distinction between individual bursts questionable.

EXAMPLE 1

COMPARISON OF THE EFFECTS OF rHu Epo AND rHu IGF-I IN THE PRESENCE OR ABSENCE OF rHu IL-3 AND HEMIN

In the initial experiments, we examined the effects of several growth factor permutations upon the of

erythroid day-14 burst-component colonies from PB MNC.
Accompanying Figure 1 presents the results graphically. On
Fig. 1, triplicate or sextuplicate determinations from one
or several experiments are expressed as Means ± S.E. FAF,
GF BSA (1%) present together with the entirety of the basal
serum-free medium components as described under "Clonal
Cell Culture" above, was unable, by itself, to support the
development of burst-component colonies (Fig. 1, bar 1) and
the same negative result was obtained when Epo was added
(Fig. 1, bar 2), confirming that the BSA preparation
employed was operationally devoid of a BPA-like activity
and that the basal serum-free medium by itself does not
support growth of primary hematopoietic cells. Hence, in
order to obtain burst-formation we would need to add an
exogenous source of BPA to the medium. The same results
showed that the recombinant Epo preparation was equally
devoid of a BPA contaminant, which is often present in
semi-purified Epo preparations.[10] This contrasted with the
results obtained when rHu Epo alone was added to Cohn's Fr.
V BSA (1%). Under these conditions 18 ± 2.5 single-colony
bursts developed per 2 x 10⁵ cells. However, the results
thus obtained with Fr. V BSA were erratic, suggesting that
this source of albumin has variable quantities of contami-
nating BPA and may, at times, have none, at least oper-
ationally.

The addition of a defined, exogenous BPA-like
activity in the form of 5.5 ng/ml of rHu IL-3 was also
unable to promote erythroid burst-formation in the absence
of any added Epo (Fig. 1, bar 3), demonstrating that the
BSA employed is also devoid of an Epo-like activity. In
fact, examination of these cultures showed the presence of
morphologically erythroid-like colonies that failed to
mature, along with granulocytic and monocytic colonies.
The addition of 0.1 mM hemin to this basal level of IL-3
did not elicit erythroid differentiation of these colonies

either (Fig. 1, bar 4), and addition of rHu Epo and rHu IL-3 showed the presence of a few day-14 erythroid colonies (or single-colony bursts) scorable only with acid benzidine (Fig. 1, bar 5). Addition of 0.1 mM hemin to the combination of Epo and IL-3 dramatically increased the colony-forming efficiency (7-fold, Fig. 1, bar 6). Under these conditions, colonies could now be scored directly in the microscope by their orange colour alone. A comparison of bars 6 and 7 in Fig. 1 shows that, in the presence of hemin and IL-3, addition of IGF-I could effectively replace Epo with respect to burst formation. However, when Epo and IGF-I were added together (at the previously established concentrations), their combined effect was greater than that obtained with either factor alone, but less than the sum of their separate effects (Fig. 1, bar 8).

EXAMPLE 2

OPTIMIZATION OF SERUM-FREE CULTURE CONDITIONS

It was reported in 1982 that some retinoids can significantly increase erythroid burst formation.[26] Hence, in order to improve the efficiency of the medium, we tested the effects of combining hemin and retinyl acetate with the full complement of recombinant factors, Epo, IL-3, and IGF-I, except for SCF. We had in this medium previously found that, by themselves, retinyl acetate and all-trans-retinoic acid also had an Epo-like activity, in that they appeared to function as differentiation factors.[27] Fig. 2 also shows that with respect to numbers of burst-component colonies, the effect of a combination of hemin and retinyl acetate (bar 4-bar 1) was significantly greater than the sum of their separate effects [(bar 2-bar 1) + (bar 3-bar 1)], suggesting a synergism, but this effect was not detectable for bursts (bar 4 - bar 1) vs [(bar 2 - bar 1) + (bar 3 - bar 1)].

Together, hemin and retinyl acetate induced a 4.8-fold increase in the number of burst-component colonies and a 3.5-fold increase in the number of bursts. This contrasts with a 2.8-fold increase in burst-component colonies and a 2.6-fold increase in bursts obtained by stimulation with retinyl acetate only. However, in the presence of serum (10% FBS) and a "dirty" BSA (Cohn's Fr. V), no synergism was apparent (Fig. 2, bars 6a and 7a for bursts, and 6b and 7b for colonies). The highest number of burst-component colonies in serum was significantly lower than that in SF medium (bar 6 vs bar 4), suggesting the presence of inhibitory factors in serum.

Similar results have been obtained with hemin together with all-_trans_ retinoic acid ATRA (Fig. 3).

To find out whether ATRA behaves in the same way as retinyl acetate, we substituted ATRA for RA (at 30nM) in the same SF medium. We found that in the absence of any added BPA-like activity (IL-3 or hemin) and of any Epo-like activity (Epo or IGF-I), ATRA was not capable by itself of supporting early colony growth and differentiation (Fig.3, bar 1). In the presence of Epo, the normal regulator of ethrythropoietic differentiation, ATRA did not provide a BPA-like activity in that no day-16 colony formation was detected (Fig. 3, bar 2). In the presence of rHu-IL-3, erythroid colonies could be morphologically recognized but no hemoglobinization could be detected (Fig. 3, bar 3). Substituting optimal concentrations of Epo and IGF-1 for ATRA (on an IL-3 background) yielded a comparable number of day-16 colonies (not significantly different from bar 5, Fig. 3; t=2.46 for 2 degrees of freedom and p >0.05) and a much stronger degree of hemoglobinization (Fig.3, bar 6); still, other erythroid-like colonies could be observed which had not matured. However, colony maturation was dramatically increased when ATRA was added to complete the

SF medium (Fig. 3, bar 7), as it induced a 4-fold increase
in the number of hemoglobinized d16 colonies. These find-
ings show that ATRA functions in a manner analogous to RA,
and strongly suggest that these retinoids act as essential
co-factors of erythroid differentiation.

EXAMPLE 3
CELL-DOSE RESPONSE IN AN IMPROVED SF MEDIUM

The relation between the number of bursts pro-
duced and the number of PB MNC plated was investigated.
The results in Fig. 4 show that, in the presence of 5.5
ng/ml of rHu IL-3, 3.0 U/ml rHu Epo, $3 \times 10^{-8}M$ IGF-I, 0.1 mM
hemin and $3 \times 10^{-8}M$ retinyl acetate, the number of bursts
varied linearly with the cell concentration between 1 and
10×10^4 cells/0.5 ml plated. The regression line relating
these variables extrapolated through a point not signifi-
cantly different from the origin, suggesting that the
medium is performing satisfactorily for burst production.
The efficiency of burst-production was approximately 1
burst/1,300/PB/MNC plated.

EXAMPLE 4
BFU-E REQUIREMENTS FOR rHu IL-3 AND HEMIN IN THE IMPROVED
SF MEDIUM

A titration of rHu IL-3 (Fig. 5) in the SF medium
showed that the number of day-14 burst-component colonies
varied linearly with the log of rHu IL-3 concentration. In
the absence of added IL-3, no bursts could be detected; the
erythroid colonies that were present had a soft orange
colour and had < 50 cells. At IL-3 concentrations of up to
0.28 ng/mL, hemoglobinized bursts were practically absent,
but burst-component colonies could still be recognized by
their morphology and a faint orange colour, even though
their number and the cellularity of each colony were very

low. Between 0.28 and 5.5 ng/ml, colony numbers increased, they had a mean cellularity of about 100 cells/colony, and a few, much larger colonies could also regularly be observed. A plateau of activity was reached at 5.5 ng/ml of rHu IL-3, without any toxic effects. From these data, we chose to use 5.5 ng/ml of rHu IL-3 as our standard concentration of added BPA-like activity for the improved SF medium.

In the improved SF medium, hemin facilitated visualization of hemoglobinized colonies by increasing the intensity of their colour: at 10 μM, hemin did not increase the number of bursts or their colonies (Fig. 6), but conferred on them a stronger hemoglobinization, their colour now being a definite orange instead of the soft, pale orange colour of the colonies present in the absence of hemin; at 100 μM hemin, the colonies became redder, and at 250 μM they seemed to attain a maximal degree of redness. Besides this qualitative effect, which greatly facilitated in situ scoring, both the number of day-14 bursts and the number of day-14 burst-component colonies increased linearly with increasing hemin concentration between 10 and 250 μM (Fig. 6). Over this range, the number of day-14 burst-component colonies increased 4-fold and the number of day-14 bursts increased 2-fold. The optimal concentration of hemin for both maximal hemoglobinization and number of day-14 bursts and their component colonies derived from 10^5 PB MNC was 250 μM. At this cell density, the 500 μM concentration of hemin was too high for purposes of colony and burst enumeration; colony growth became practically confluent as early as day-12, and by day-14 some lysis was detectable. Still higher concentrations tested (1 mM) were toxic and resulted in widespread lysis of cells in the colonies.

EXAMPLE 5

TITRATION OF rHu IGF-I IN THE ABSENCE OF Epo, WITH AND
WITHOUT RETINYL ACETATE

Next, we titrated the number of burst-component
colonies against rHu IGF-I concentration, in the absence of
Epo or retinyl acetate (Fig. 7). At low concentrations
($<10^{-10}$M), the number of erythroid burst-component colonies
was significantly lower than would have been expected if we
extrapolated back the linear component of the response
curve between 10^{-10} and 3×10^{-10}M IGF-I (Fig. 7, lower curve,
closed squares). This suggests that a threshold concentra-
tion of IGF-I may be necessary for erythroid burst-compo-
nent colony formation to be detected. Titration of burst-
component colonies against rHu IGF-I concentration, using
the same PB MNC but performed in the presence of retinyl
acetate (also without Epo), showed that the effect of IGF-I
was proportional to the log of its molarity, between 3×10^{-11}
and 3×10^{-8}M, without having reached a plateau of activity
at the highest concentration tested (Fig. 7, upper curve,
closed circles). Accordingly, the half-maximal stimulation
must be $> 3 \times 10^{-10}$M (>2.6 ng/ml). At 3×10^{-11}M, rHu IGF-I
appeared to be ineffective against a background of early
erythroid colonies which were apparently promoted by the
presence of the retinoid at a concentration of 3×10^{-8}M.
Once again, retinyl acetate at the highest effective
concentration greatly enhanced ($\sim$ 5-fold) the number of
burst-component colonies scored.

EXAMPLE 6

COMPARISON OF THE TITRATIONS OF rHu Epo AND rHu IGF-I UNDER
OPTIMAL SF CONDITIONS FOR BURST-PRODUCTION

The effects of either Epo or IGF-I on the produc-
tion of burst-component colonies, each titrated in the
improved SF medium, but in the absence of the other GF,

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were compared.   The results are expressed as raw data
(Figs. 8A and 8B) and as percentage of the maximal stimula-
tion observed with each GF (Fig. 8C).   The Epo dose-
response curve (continuous line joining the closed circles)
is the mean of 3 separate experiments from different
donors.   Epo in our medium showed the familiar type of
dose-response curve, plateauing at 3-6 U/ml or 9-18 x 10^{-10}M
(Epo mol. wt. 34,000).   Below 2 x 10^{-11}M rHu Epo, the number
of burst-component colonies lay within the background level
induced by retinyl acetate alone, in the absence of any Epo
or IGF-I.   From this normalized Epo curve, the half-maximal
stimulation of the hormone upon burst-component colony
formation was 2.7 x 10^{-10}M (0.89 U/ml), which corresponds to
an Epo protein concentration of 8.1 ng/ml.

A rHu IGF-I titration from 2 experiments with
different donors is also shown in Fig. 8B (one in closed
circles, the other in open squares).   Each is expressed
both as raw data (Fig. 8B) and as a percentage of its own
maximal effect (Fig. 8C).   It is apparent that the concen-
tration of rHu IGF-I needed to reach maximal activity (at
10^{-7}M) is nearly two logs of molarity higher than what we
have observed with Epo in the same medium.   Between 3 x 10^{-11}
and 3 x 10^{-7}M rHu IGF-I, the number of burst-component
colonies varied proportionately with the log of IGF-I
concentration, its half-maximal stimulation lying around
6.5 x 10^{-10}M IGF-I, which corresponds to a protein concentra-
tion of 5.6 ng/ml. It is worth remarking that the overall
numbers of burst-component colonies obtained by stimulation
with each growth factor alone, as seen at plateau concen-
trations, were not significantly different from one another
(a range of 462 to 503 colonies/10^5 MNC for all 5 experi-
ments).

Titration of rHu Epo and rHu IGF-I expressed in
terms of the number of bursts produced (Fig. 9A) and as a

percentage of their maximal effect (Fig. 9B) showed that
the differential sensitivities to the respective growth
factors were approximately the same as those observed for
burst-component colonies, both for the proportional and the
plateau components of the curves. From the actual numbers
obtained in all these experiments we calculated the number
of burst-component colonies per burst both at the back-
ground level of retinoids (ie in the absence of both IGF-I
and Epo, or at their ineffective concentrations), and at
their plateau levels. The value of background averaged 1.7
colonies (with > 50 cells each) per burst (77 colonies per
42 bursts/10^5 MNC); the values at plateau averaged 4
colonies per burst (467 colonies per 116 bursts) at maximal
IGF-I stimulation and 5.4 colonies per burst (488 colonies
per 91 bursts) at maximal Epo stimulation. This shows that
one of the actions common to both growth factors is to
enhance the proliferation of BFU-E-derived progenitors
within each burst.

These experiments showed, under optimal condi-
tions, that IGF-I can substitute for Epo in the production
of erythroid bursts. An investigation was conducted to
determine whether erythroid bursts would develop under the
influence of IGF-I in the presence of antibody directed
against Epo. In Fig. 10, bar 1 shows the number of bursts
obtained in the full SF medium but lacking IGF-I, with 6.0
U/ml of Epo. Comparison with bar 4, in which anti-Epo
antibody was added to the same medium containing Epo, shows
that the antibody was effective in inhibiting burst forma-
tion that was dependent on Epo. Bar 3 shows that burst
formation under the influence of IGF-I was not affected by
the same anti-Epo antibody. Similar results were obtained
when the day-14 cultures were scored for burst-component
colonies. This conclusively demonstrates that the produc-
tion of erythroid bursts under the influence of IGF-I does
not occur through an Epo-dependent mechanism. Bars 4 and

5 show that in this SF medium a limited number of bursts form which do not require either Epo or IGF-I, and the anti-Epo antibody has no effect on their development. Evidently, the presence of IL-3, hemin and retinyl acetate or all-_trans_-retinoic acid is sufficient to support the production of these "Epo- and IGF-I-independent bursts".

EXAMPLE 8
ROLE OF ACCESSORY CELLS IN BURST PRODUCTION IN AN IMPROVED SF MEDIUM

The experiments described thus far were done with Ficoll-Hypaque density gradient-separated cells, and they provided information on the growth factor requirements of human erythroid progenitors among PB MNC, against a SF background. To find out whether or not accessory cells play a role under these conditions, we removed plastic adherent cells. Fig. 11 shows that production of erythroid bursts could be completely eliminated by this procedure, despite the presence of IL-3, hemin, retinyl acetate, Epo and IGF-I at what would otherwise be optimal concentrations. Thus accessory cells clearly still play a crucial role in the production of erythroid bursts by PB MNC in the improved SF medium.

EXAMPLE 9 - EFFECT OF RECOMBINANT HUMAN STEM CELL GROWTH FACTOR AS A REPLACEMENT FOR ADHERENT CELLS

Recombinant human (rHu) SCF was added to the SF cultures of Ficoll-Hypaque density gradient separated MNC which had been exposed to 1.5 hours' adherence to plastic.

When the rHu SCF was added to cultures of untreated PB MNC under SF conditions at concentrations ranging from 30 to 1500 pM (1 to 50 ng/mL) in the absence of IL-3, they showed virtually no change in the number of

bursts (Fig. 12A closed circles) or of burst-component colonies (BCC) (Fig. 12B, closed circles). In the presence of 0.8 nM rHu IL-3, no significant increase (t=2, with 2 df and p>0.1) in burst-formation was observed at 1.5 nM rHu SCF (Fig. 12A open circles). In contrast, adherent cell-depleted PB MNC showed a significant increase of burst- and BCC-formation upon the addition of as little as 30 pM rHu SCF (Figs. 12A and 12B, closed squares), but no increase upon the addition of IL-3 (in the presence of hemin and 1.5 or 3.0 nM rHu SCF). The control point shown in parentheses (closed squares at 0 rHu SCF in Fig. 12B) represents erythroid colonies with <50 cells (an average of 25) which are promoted by the BPA-like action of hemin alone and were tentatively grouped as clusters, possibly equivalent to undeveloped bursts (Fig. 12A, closed square in parentheses). Some of these small hemin-dependent colonies approached the 50-cell size, and the effect of rHu SCF, under these conditions (in the absence of IL-3), was to increase the cellularity (up to 200 cells) of the BCC; it also rendered the colonies rounder and more compact. It thus allowed full burst development.

A comparison of the effects of rHu SCF and of the two defined BPA-like activities used in our SF medium (18), hemin and rHu IL-3, upon burst-formation shows that optimal conditions for burst-formation appeared to require the presence of all three factors, rHu IL-3, rHu SCF and hemin (Fig. 12C, open bar 3), even though the addition of rHu IL-3 to both hemin and rHu SCF did not significantly increase the number of BCC observed (cf open bars 2 and 3, Fig. 12D). Removal of adherent cells appeared to prevent the capacity of hemin to promote erythroid colonies large enough for them to be classified as BCC; the number of erythroid colonies with 20 to 50 hemoglobinized cells (Fig. 12D, striped bar 1) and of "undeveloped bursts" (Fig. 12C, striped bar 1) were fewer than the number of BCC and bursts

formed in the absence of hemin (Figs. 12C and 12D, closed
bars 4). Finally, addition of rHu IL-3 to a combination of
hemin and rHu SCF did not significantly increase the number
of bursts obtained with hemin and rHu SCF alone (cf closed
bars 2 and 3, Fig. 12C) and only marginally increased the
number of BCC obtained with hemin and rHu SCF (cf closed
bars 2 and 3, Fig. 12D).

EXAMPLE 10

EFFECT OF PROLIFERATION-PROMOTING GROWTH FACTORS (Hemin,
rHu-IL-3 and rHu SCF) UPON MULTI-LINEAGE HEMATOPOIETIC
COLONIES FROM HUMAN BONE-MARROW

Normal bone-marrow mononuclear cells (BM MNC) separated and
washed as described in Materials and Methods for PB MNC
were grown in the basal serum-free medium, together with
3.0 μ/ml of rHu Epo, 3×10^{-8}M rHu IGF-1 and 3×10^{-8} retinyl
acetate, with different combinations of proliferation-
stimulatory activities (250 μM Hemin, 10 ng/ml rHu IL-3 and
50 ng/ml rHu SCF). Hemin is necessary to detect both
erythroid (CFU-E- and BFU-E-derived) and non-erythroid
(CFU-GM-derived) colonies (Fig. 13 bars 1), but the numbers
of these colonies are significantly increased by the
addition of rHu IL-3 (bars 2). The addition of rHu SCF in
the absence of rHu IL-3 further increased the levels of
these colonies over those obtained with rHu IL-3 in the
absence of rHu SCF (CP. bars 3 and 2), and expression of
multi-lineage CFU-GEMM-derived colonies could be observed
under these conditions (Cp. open bar 3). Lastly, optimal
levels of all four types of colonies were obtained when
Hemin, rHu IL-3 and rHu SCF were all added together, making
the medium complete (bars 4).

          Results shown in Fig. 14 were obtained using
human bone-marrow mononuclear progenitor cells from

patients with Diamond Blackfan Anemia (DBA). Bars 1 of
Fig. 14 are comparable to bars 1 of Fig. 13 as obtained
under the same culture conditions (complete medium minus
rHu IL-3 and rHu SCF). Bars 2 of Fig. 14, however, show
that DBA patients have dramatically fewer CFU-E (late
erythroid progenitors) and BFU-E (early erythroid progeni-
tors) than normals do (CP bars 3 of Fig. 13) when rHu SCF
is added to Hemin in the absence of rHu IL-3. Finally, the
addition of rHu IL-3 to Hemin in the absence of rHu SCF
(Fig. 14, bars 3) exhibited the same greatly diminished
expression of erythroid bone-marrow progenitors in DBA but
it also indicates a greater expression of non-erythroid
(CFU-GM) progenitors in DBA than in normals (CP. open bar
3 from Fig. 14 with the more densely stippled bar 2 of Fig.
13).

EXAMPLE 11

ERYTHROID COLONY FORMATION BY POLYCYTHEMIA VERA PRIMARY AND
SECONDARY CELL POPULATIONS IN AN IMPROVED SF MEDIUM

Fig. 15 shows the cell-dose response curve for
burst-formation by PB MNC from a Polycythemia vera (PV)
patient in our novel SF medium, without rHu SCF (closed
circles) and without both rHu SCF and rHu Epo (stippled
circles). In both instances, the medium contained 10 ng/ml
rHu IL-3, 0.25 mM Hemin, 3×10^{-8}M retinyl acetate and 3×10^{-8}M
rHu IGF-1. Where indicated rHu Epo was employed at 3 U/ml.
Either curve of Fig. 15 extrapolates through points not
significantly different from the origin, indicating that,
with or without rHu Epo, our novel SF medium is performing
satisfactorily for burst-formation by PV PB MNC.

The improved SF medium performs equally well for
an analysis of "CFU-E-like" and "BFU-E-like" erythroid
colonies obtained from an immortalized cell line isolated

from the peripheral blood of another PV patient, as is shown by the two curves of Fig. 16, one for day 7 erythrocytic-like colonies (open circles) and the other for day-14 burst-like colonies (closed circles).

EXAMPLE 12

AUTOCRINE GROWTH OF A BI-PHENOTYPIC LEUKEMIA CELL LINE IN SF MEDIUM

A bi-phenotypic leukemia cell line (B-1) from a patient with ALL (Acute Lymphoblastic Leukemia) was studied for its growth characteristics in our novel SF medium. Growth could be detected with the basal SF medium alone (in the absence of all peptide growth factors, namely rHu IL-3, rHu SCF, rHu EPO and rHu IGF-1, as well as in the absence of Hemin and retinoids), as shown in bar 2 of Fig. 17A. Substraction of d-α-tocopherol from the basal SF medium significantly diminished the viability of those bio-phenotypic leukemic cell day-9 colonies (bar 1, Fig. 17A) whereas addition of retinyl acetate further increased their growth (bar 3, Fig. 17A). These results indicate that B-1 cells can grow in the basal SF medium alone, in the absence of any growth-stimulating activities, presumably by means of an autocrine mechanism.

It is likely that this mechanism involves the secretion of IGF-1 by these cells, inasmuch as the addition of IGF-1 alone to the basal SF medium nearly tripled the number of B-1 colonies (CP. bars 2 and 1 of Fig. 17B).

REFERENCES

1. Kanamura A, Okamoto T, Hara H, Nagai K: Developmental Changes in Erythropoietin Responsiveness of Late Erythroid Precursors in Mouse Hemopoietic Organs. Dev Biol 92:221, 1982

2. Beckman B, Belegu RD, Belegu M, Katsuoka Y, Fisher JW: Hypoxic Enhancement of Murine Erythroid Colony Formation, in Baum, Ledney, Thierfelder (eds): Experimental Hematology Today, New York, S. Karger, 1982, p 53.

3. Kurtz A, Hart W, Jelkmann W, Zapf J, Bauer C: Activity in Fetal Bovine Serum That Stimulates Erythroid Colony Formation in Fetal Mouse Livers is Insulin-like Growth Factor Int J Clin Invest 76:1643, 1985

4. Kurtz A, Jelkmann W, Bauer C: A New Candidate For The Regulation of Erythropoiesis: Insulin-like Growth Factor I. FEBS 149:105, 1982.

5. Akahane K, Tojo A, Urabe A, Takaku F: Pure Erythropoietic Colony and Burst Formations in Serum-Free Culture and Their Enhancement By Insulin-like Growth Factor I. Exp Hematol 15:197, 1987.

6. Stewart S, Zhu B-D, Axelrad AA: A "Serum-Free" Medium For The Production of Erythropoietic Bursts By Murine Bone Marrow Cells. Exp Hematol 12:309, 1984.

7. Dainiak N, Kreczko S, Cohen A, Pannell R, Lawler J: Primary Human Marrow Cultures For Erythroid

Bursts in Serum-Substituted Culture. Exp Hematol
12:309, 1985.

8.   Porter PN, Ogawa M: Erythroid Burst-Promoting
        Activity (BPA), in Dunn CDR (ed): Current Con-
        cepts in Erythropoiesis, New York, John Wiley &
        Sons Ltd., 1983, p 81.

9.   Sonoda Y, Yang Y-C, Wong GG, Clark SC, Ogawa M:
        Erythroid Burst-promoting Activity of Purified
        Recombinant Human GM-CSF and Interleukin-3:
        Studies With Anti-GM-CSF and Anti-IL-3 Sera and
        Studies in Serum-Free Cultures. Blood 72:1381,
        1988.

10.   Konwalinka G, Geissler D, Peschel C, Breier C,
        Grunewald K, Odavic R, Braunsteiner H: Human
        Erythropoiesis In Vitro And The Source of Burst-
        Promoting Activity in a Serum-Free System. Exp
        Hematol 14:899, 1986.

11.   Migliaccio G, Migliaccio AR: Cloning of Human
        Erythroid Progenitors (BFU-E) in the Absence of
        Total Bovine Serum. Br J Haematol 67:129, 1987.

12.   Migliaccio G, Migliaccio AR, Adamson JW: In
        Vitro Differentiation of Human Granulocyte/
        Macrophage and Erythroid Progenitors: Compara-
        tive Analysis of the Influence of Recombinant
        Human Erythropoietin, G-CSF, GM-CSF, and IL-3 In
        Serum-Supplemented and Serum-Deprived Cultures.
        Blood 72:248, 1988.

13.   Misago M, Chiba S, Kikuchi M, Tsukada J, Sato T,
        Oda S, Eto S: Effect of Recombinant Human
        Interleukin 3, Granulocyte-Macrophage Colony-

Stimulating Factor and Granulocyte Colony-Stimu-
lating Factor on Human BFU-E In Serum-Free Cul-
tures. Intl J Cell Cloning 7:37, 1989

14.   Konwalinka G, Breier C, Geissler D, Peschel C,
       Wiedermann CJ, Patsch J, Braunsteiner H: Prolif-
       eration   and   Differentiation   of   Human
       Erythropoiesis In Vitro: Effect of Different
       Lipoprotein Species. Exp Hematol 16:125, 1988.

15.   Porter PN, Ogawa M: Characterization of Human
       Erythroid Burst-Promoting Activity Derived From
       Bone Marrow Conditioned Media.   Blood 59:1207,
       1982.

16.   Linch DC, Lipton JM, Nathan DG: Identification
       of Three Accessory Cell Populations in Human Bone
       Marrow With Erythroid Burst-Promoting Properties.
       J Clin Invest 75:1278, 1985.

17.   Dukes PP, Ma A, Clemons GK, Meytes D: Measure-
       ment of Human Erythroid Burst-Promoting Activity
       By a Specific Cell Culture Assay. Exp. Hematol
       13:59, 1985.

18.   Zsebo K, Martin F, Suggs S, Wypych J, Lu H,
       McNiece I et al (1990) Biological Characteriz-
       ation of a Unique Early Acting Hematopoietic
       Growth Factor. Exp Hematol 18:703 (abs).

19.   Broxmeyer HE, Cooper S, Lyman SD, Williams DE
       (1990) Characteristics of a Murine Ligand for C-
       kit (MGF):   A Stimulating/Enhancing Factor For
       Early Murine and Human Bone Marrow Hemopoietic
       Progenitor Cells. Blood 76:134a (abs).

20.   Migliaccio G, Migliaccio AR, Valinsky K et al.
       (1990) Recombinant Rat Stem Cell Factor (rr SCF)
       Induces Proliferation and Differentiation of
       Primitive Hematopoietic Progenitor Cells (HPC) in
       Serum-Deprived Cultures. Blood 76:156a (abs).

21.   McNiece I, Langley K, Zsebo K (1991) Recombinant
       Human Stem Cell Factor Synergises With GM-CSF, G-
       CSF, IL-3 and Epo to Stimulate Human Progenitor
       Cells of the Myeloid and Erythroid Lineages. Exp
       Hematol 19:226.

22.   Anderson DM, Lyman SD, Baird A et al. (1990)
       Molecular Cloning of Mast Cell Growth Factor, A
       Hematopoietin That is Active in Both Membrane
       Bound and Soluble Forms. Cell 63:235.

23.   Brox A, Congote F, Fauser AA: A Novel Peptide
       Stimulates In Vivo and In Vitro Erythropoiesis.
       Blood 74:14a, 1989 (abstr.).

24.   DeLeenher AP, Lambert WC, Claeys I: All-trans-
       retinoic Acid: Measurement of Reference Values
       in Human Serum By High Performance Liquid Chroma-
       tography. J Lipid Res 23:1362, 1982.

25.   DeRuyter MG, Lambert WE, DeLeenher AP: Retinoic
       Acid: An Endogenous Compound of Human Blood.
       Unequivocal Demonstration of Retinoic Acid in
       Normal Physiological Conditions. Anal Biochem
       98:402, 1979.

26.   Douer D, Koeffler HP: Retinoic Acid Enhances
       Growth of Human Early Erythroid Progenitor Cells
       In Vitro. J. Clin. Invest G9:1039, 1982.

- 38 -

27.   Correa, P.N., Axelrad, A.A.: Retinyl Acetate and
        All-Trans Retinoic Acid Enhance Erythroid Colony
        Formulation By Circulating Human Progenitors in
        a Complete Serum Free Medium. Submitted for
        Publication.

WHAT WE CLAIM IS:

1.   A basal serum-free medium for erythroid and other
hematopoietic progenitor cells and leukemia cells, consist-
ing essentially of:

         a minimum essential medium;

         an effective amount of each of the four deoxyri-
bonucleosides adenine deoxyriboside, thymine deoxyriboside,
guanine deoxyriboside and cytosine deoxyriboside;

         an effective amount of each of the four ribonu-
cleosides adenine riboside, uridine riboside, guanine
riboside and cytosine riboside;

         an effective amount of L-glutamine;

         an effective amount of deionized, fatty-acid free
and globulin-free bovine or human serum albumin or recombi-
nant human albumin;

         an effective amount of a human or bovine
transferrin;

         an effective amount of a phosphatidyl choline;

         an effective amount of a C_{16} - C_{24} unsaturated
fatty acid;

         an effective amount of a cholesterol;

         an effective amount of d-α-tocopherol or an ester
thereof;

         an effective amount of at least one bio-accept-
able antioxidant;

         and an effective amount of at least one anti-
biotic substance effective to protect the cells against
stray infections.

2.   The basal serum-free medium of claim 1 wherein
the phosphatidyl choline is L-α-phosphatidyl choline
dipalmitoyl synthetic.

3.   The basal serum-free medium of claim 2 wherein
the unsaturated fatty acid is linoleic acid or oleic acid.

4.   The basal serum-free medium of claim 3 wherein
the cholesterol component is porcine liver cholesterol.

5.   The basal serum-free medium of claim 4 wherein
the antioxidant is selected from the group consisting of β-
mercaptoethanol, α-thioglycerol and combinations thereof.

6.   The basal serum-free medium of claim 5 wherein
the antibiotic substance is selected from the group con-
sisting of sodium penicillin G, streptomycin sulphate and
combinations thereof.

7.   The basal serum-free medium of claim 6 further
including an effective amount of a bio-acceptable semi-
solid or viscous matrix material.

8.   The basal serum-free medium of claim 7 wherein
the matrix material is selected from the group consisting
of methylcellulose, agar and agarose.

9.   A serum-free culture medium for the growth and
differentiation of erythroid and other hematopoietic
progenitor cells and leukemia cells, said medium being free
from indigenous cell growth factors but adapted to receive
extraneous cell growth influencing materials for determina-
tion of the effects thereof on the growth and differenti-
ation of erythroid, other hematopoietic cells, and leukemia
cells and for attaining optimal growth thereof, said
culture medium consisting essentially of:

          - a basal serum-free medium as defined above;
          - an effective amount of at least one growth
promoting agent selected from the group consisting of heme

or hemin, interleukin-3, and recombinant human stem cell factor;

- an effective amount of at least one cell differentiation-promoting agent selected from the group consisting of erythropoietin, insulin-like growth factor, and a retinoid.

10.   The culture medium of claim 9 which additionally includes an effective amount of erythropoietin.

11.   The culture medium of claim 10 which additionally includes an effective amount of interleukin-3.

12.   The culture medium of claim 11 which additionally includes an effective amount of recombinant human stem cell factor.

13.   The culture medium of claim 12 which includes an effective amount of the insulin-like growth factor IGF-1.

14.   The culture medium of claim 13 which includes an effective amount of hemin and is substantially free from heme.

15.   The culture medium of claim 14 which includes a retinoid selected from the group consisting of retinoic acid and lower alkyl esters of retinoic acid.

16.   The culture medium of claim 15 wherein the retinoid is retinyl acetate.

17.   The culture medium of claim 15 wherein the retinoid is all-_trans_ retinoic acid.

18.   The culture medium of claim 17 wherein the basal serum-free medium is as defined in claim 6.

19.   The culture medium of claim 17 wherein the basal
serum-free medium is as defined in claim 8.

20.   A process of determining the effect of a test
substance on the growth characteristics of erythroid and
other hematopoietic progenitor cells or leukemia cells
which comprises culturing said cells under predetermined,
controlled conditions in a serum-free culture medium as
defined in claim 9 and to which has been added a predeter-
mined quantity of said test substance, and recording growth
characteristics of said cells in the culture medium.

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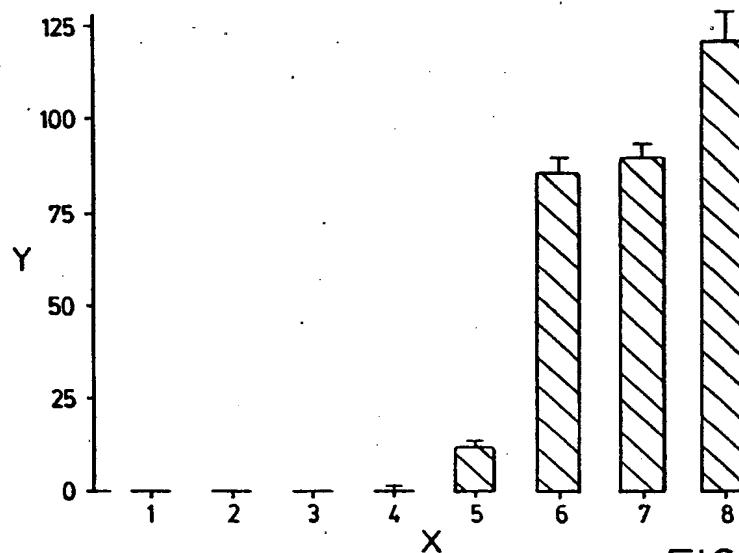
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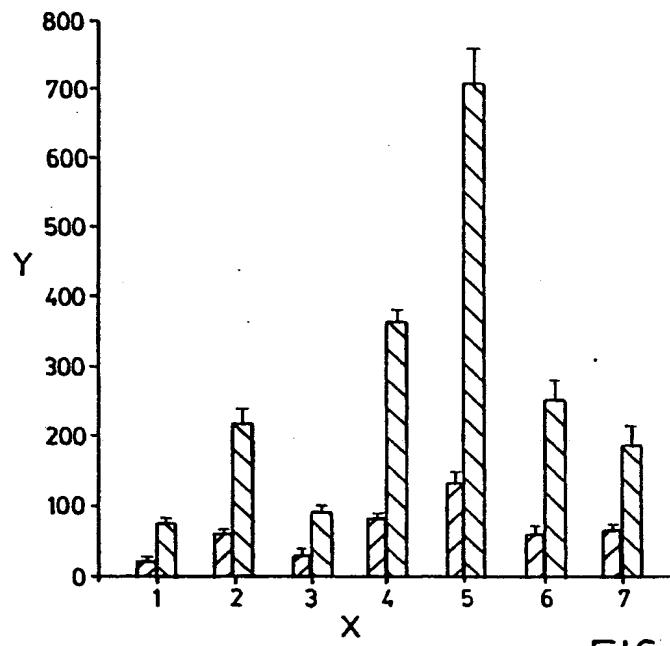
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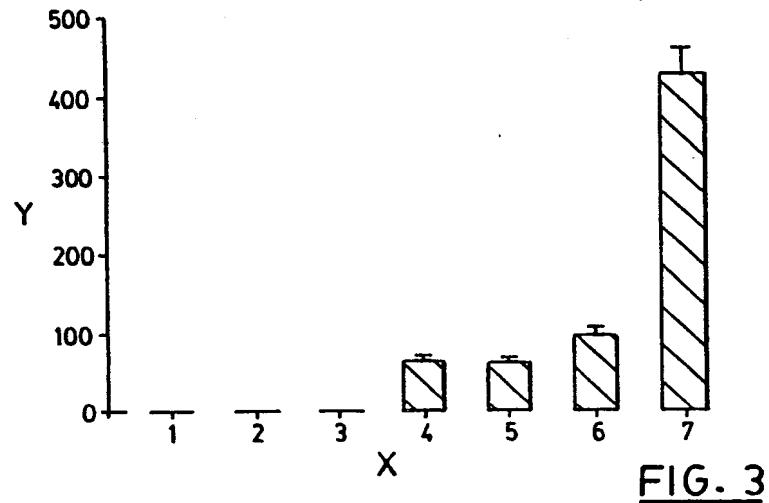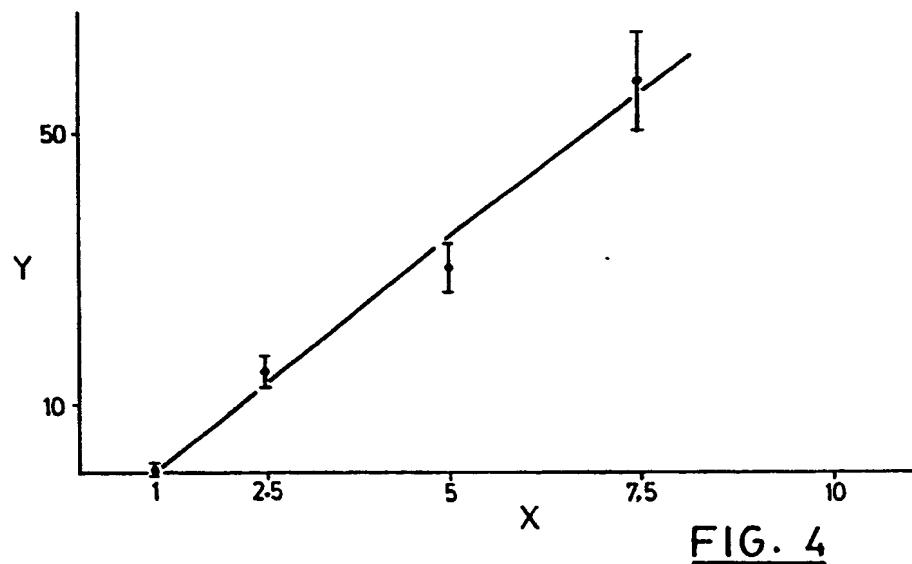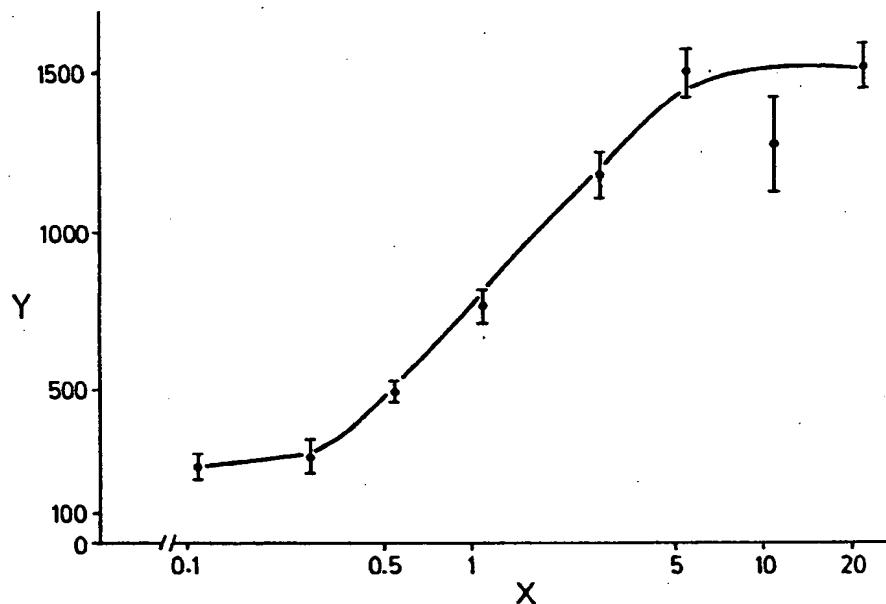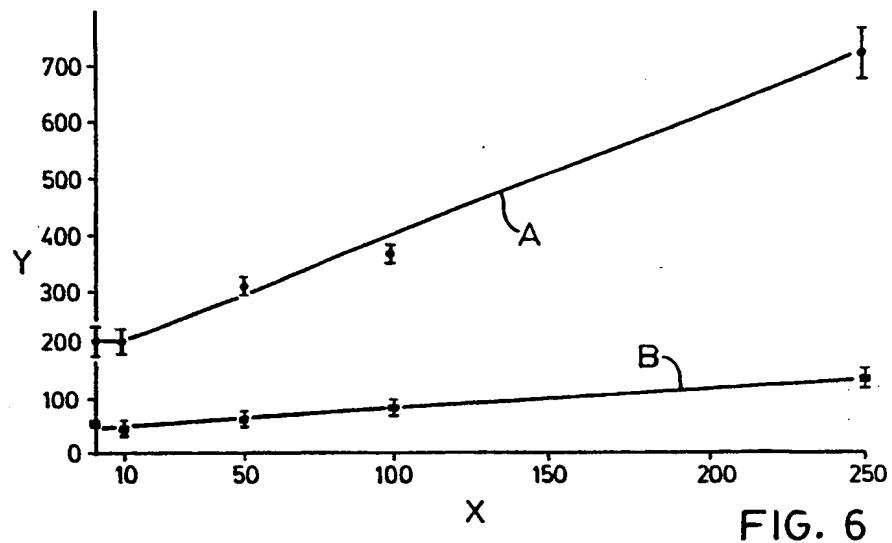
FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7

FIG. 8A

FIG. 8B

FIG. 8C

FIG. 9A

FIG. 9B

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FIG. 10

FIG. 11

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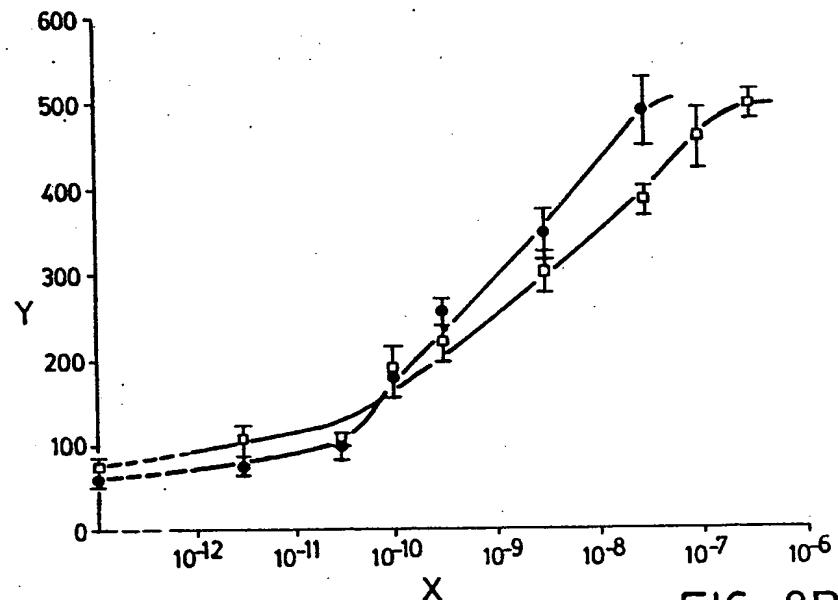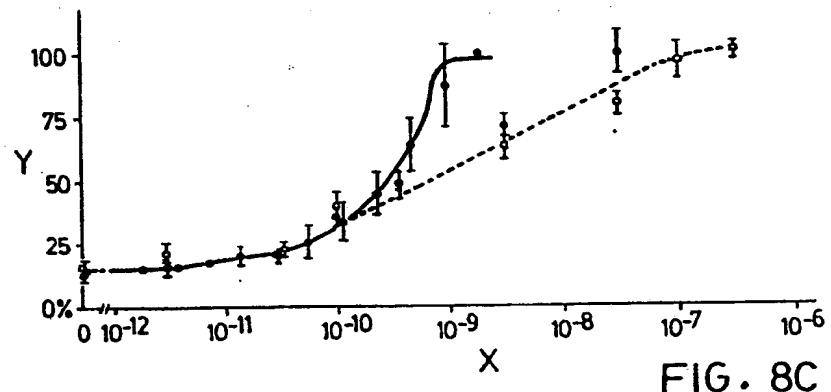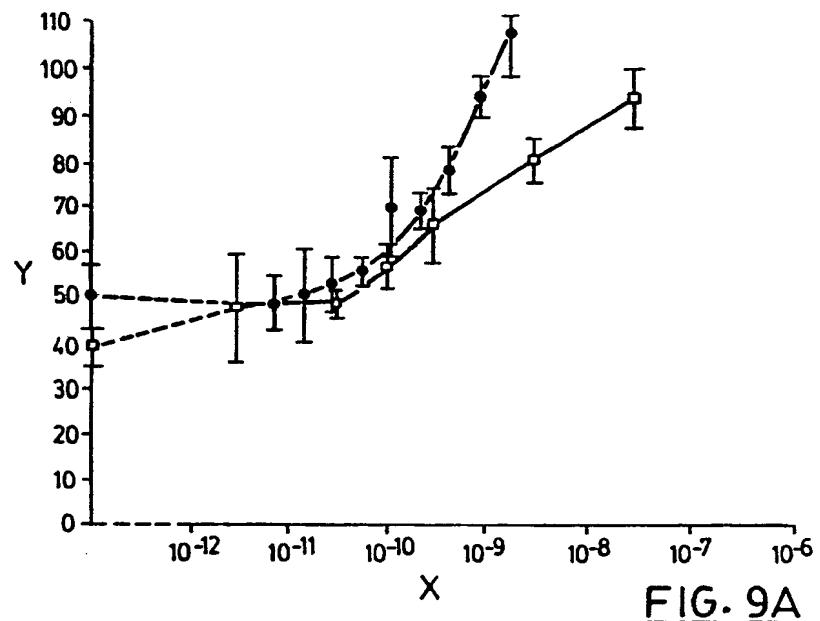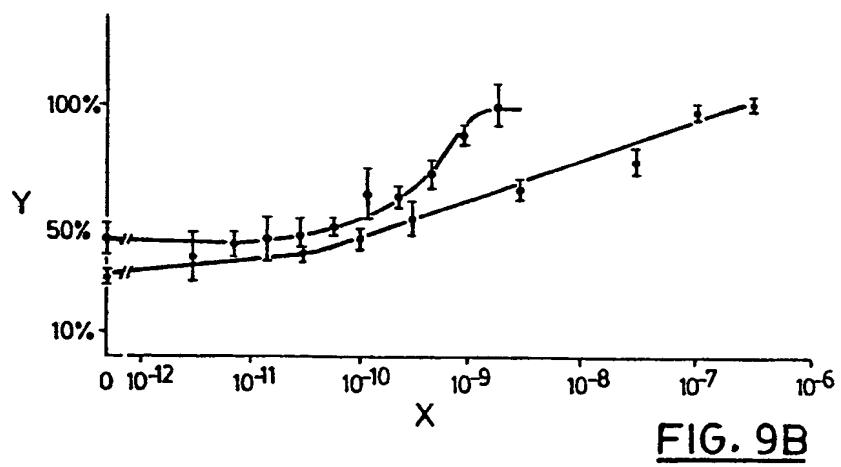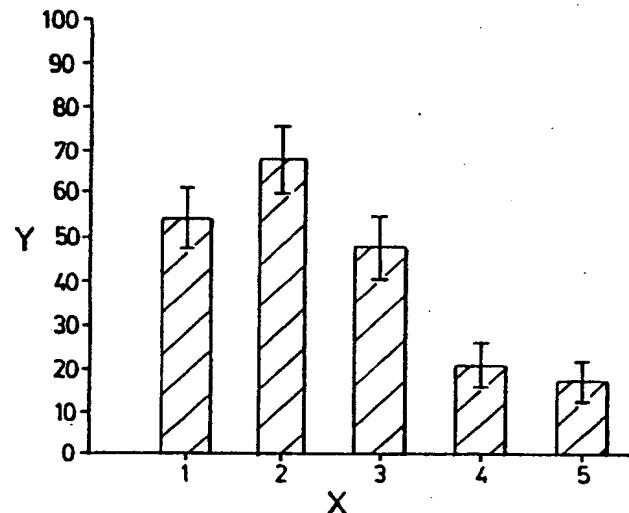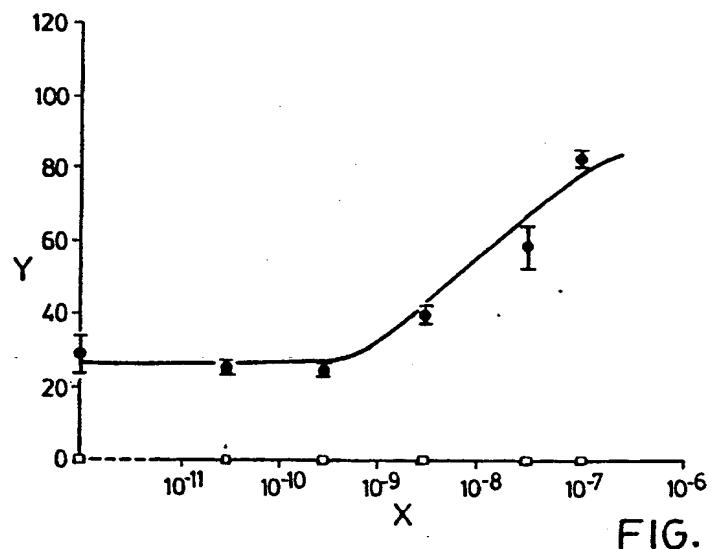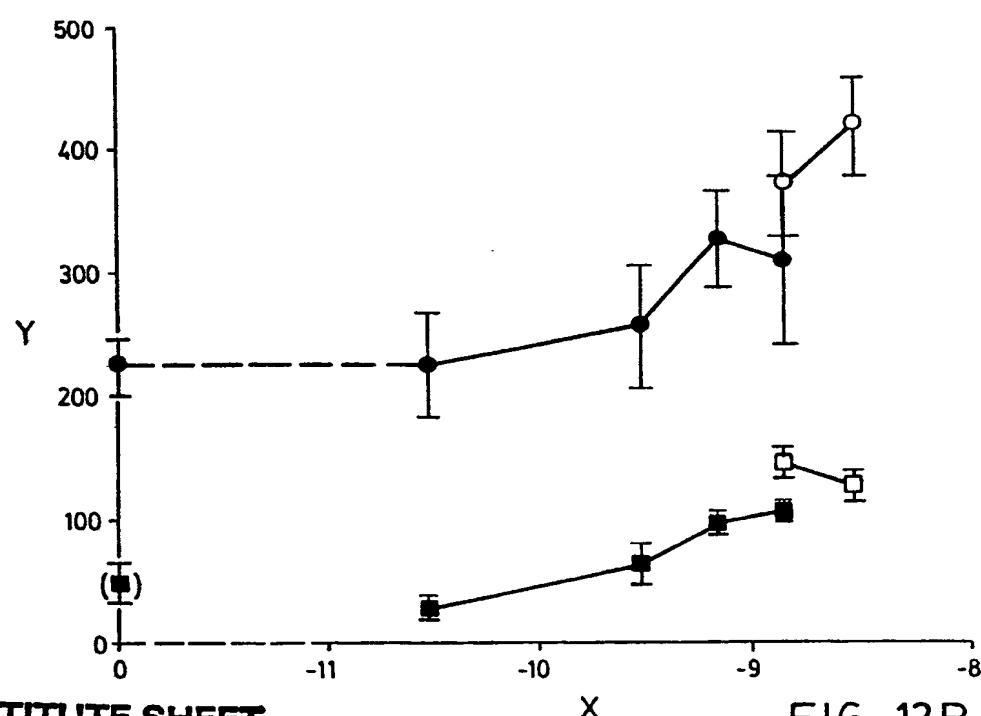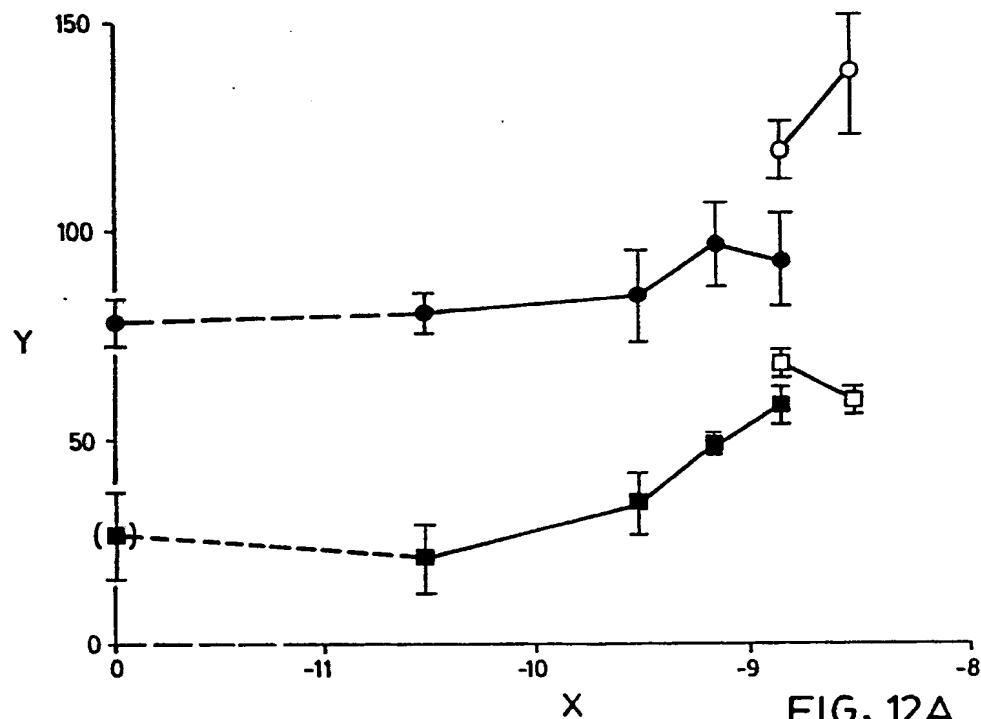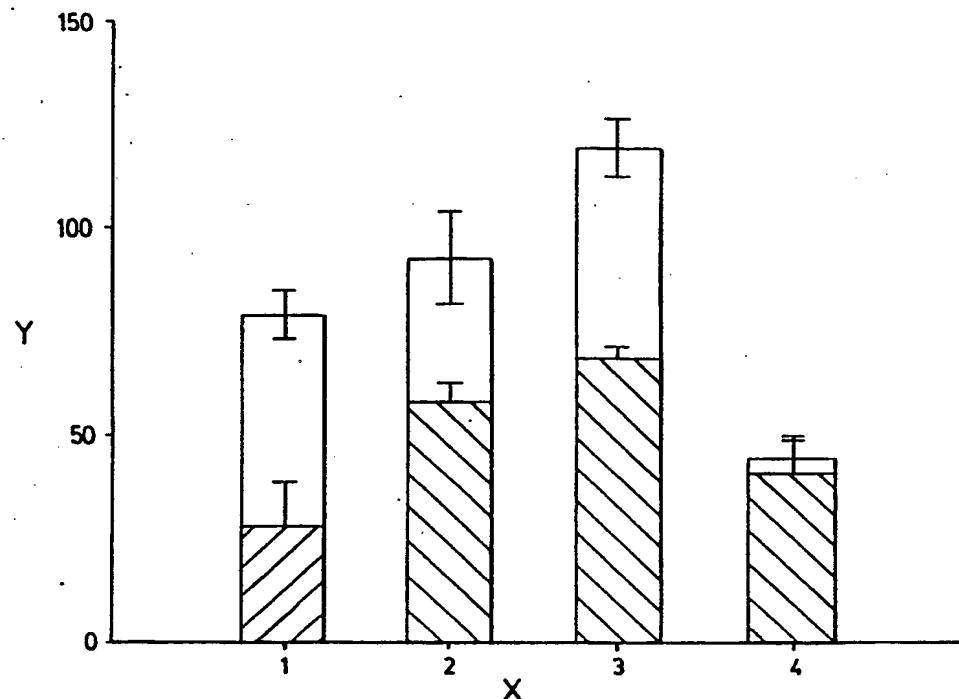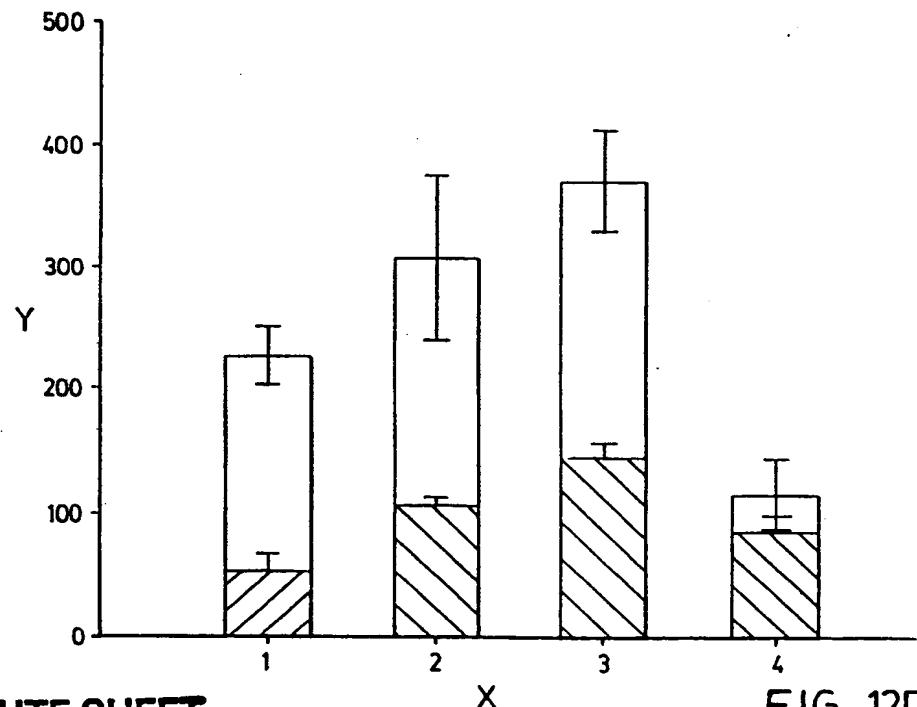
FIG. 12A

FIG. 12B

FIG. 12C

FIG. 12D

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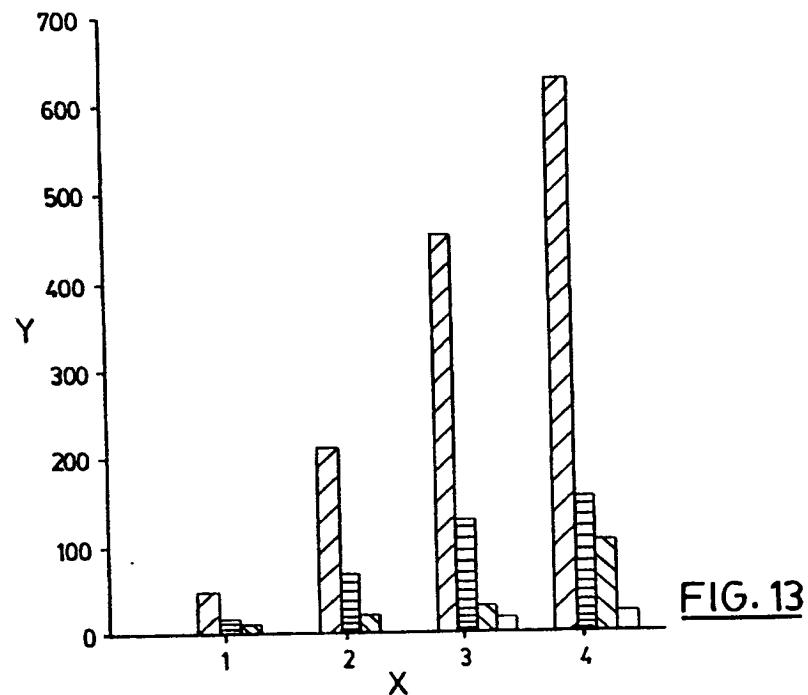
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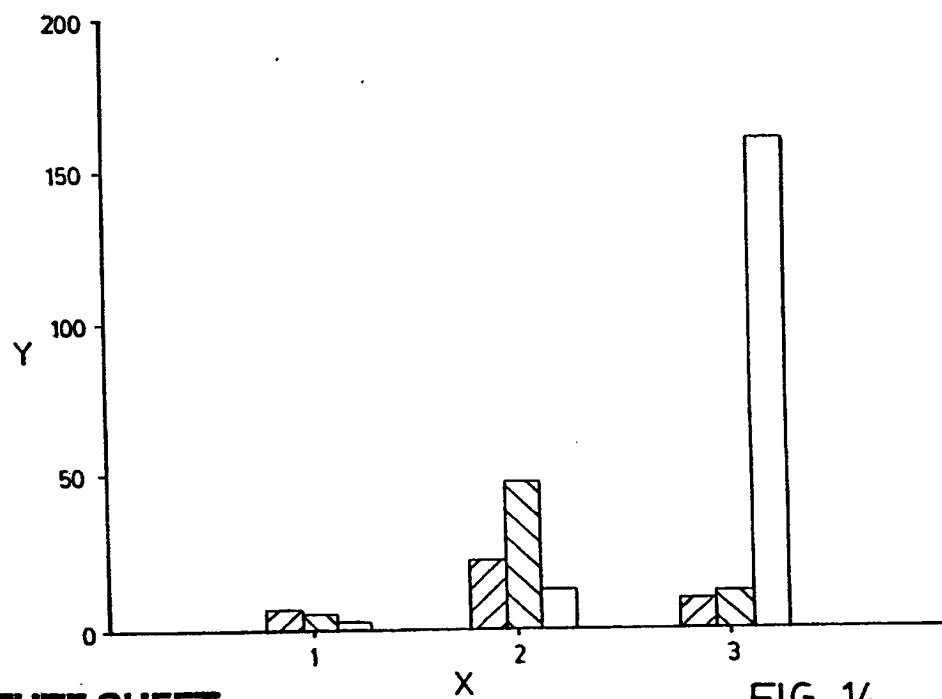
FIG. 14

11/12
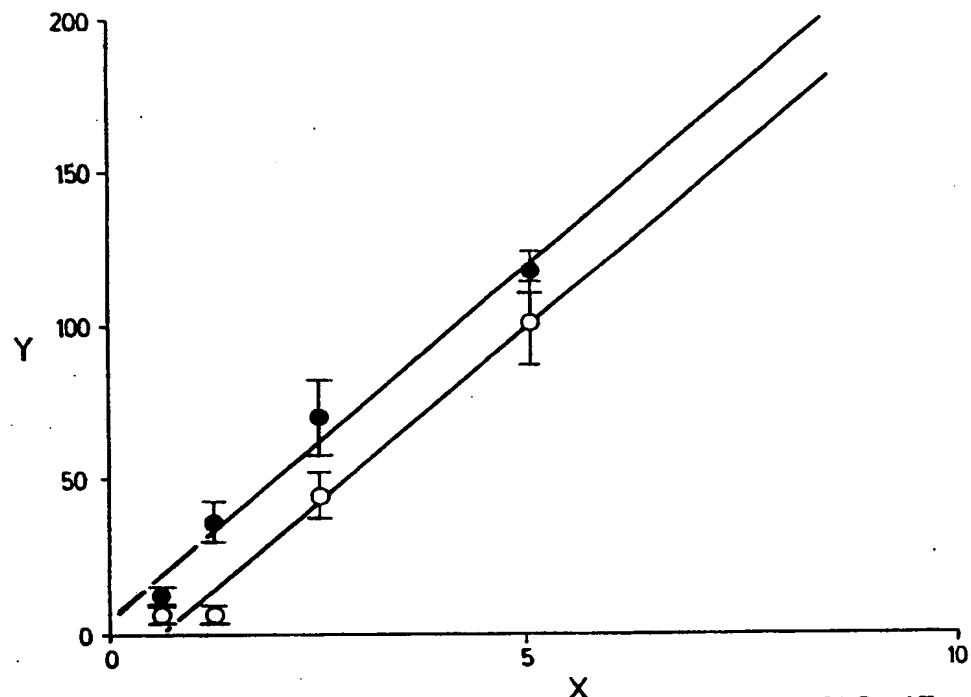

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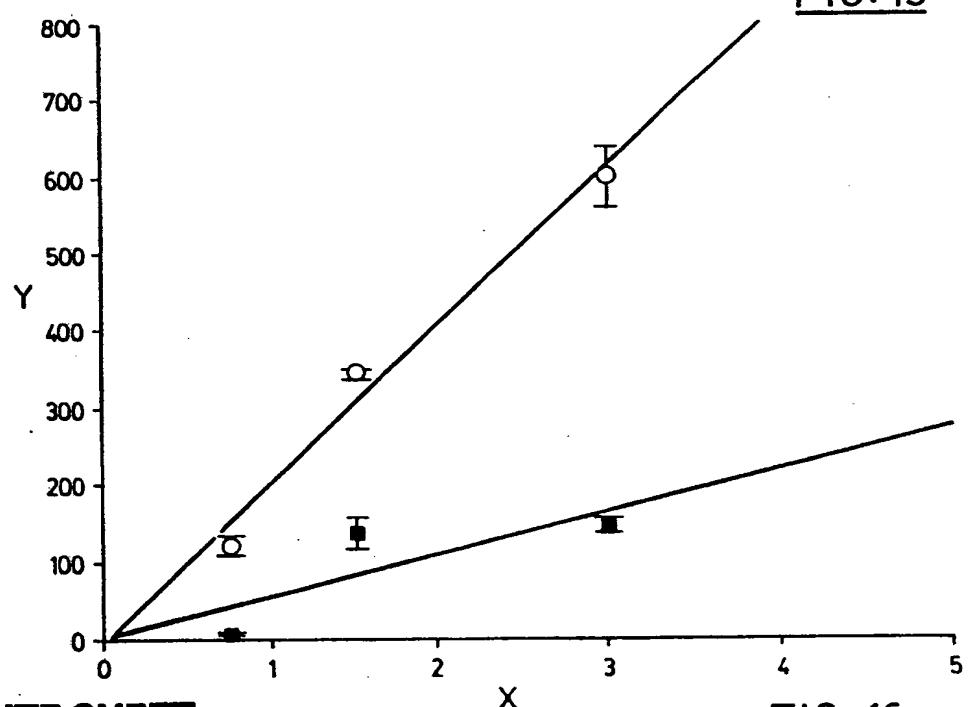

FIG. 16

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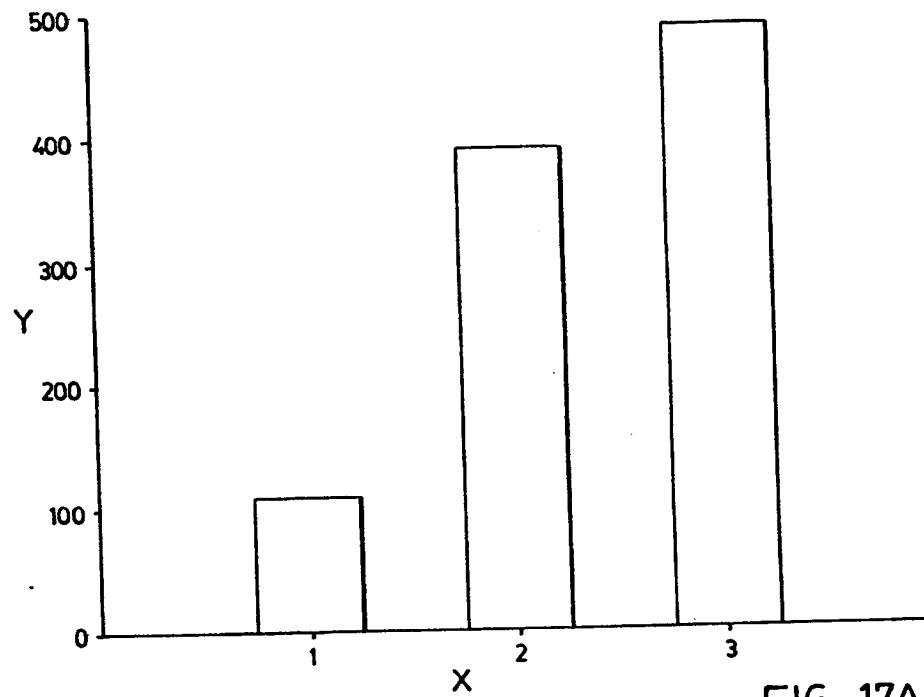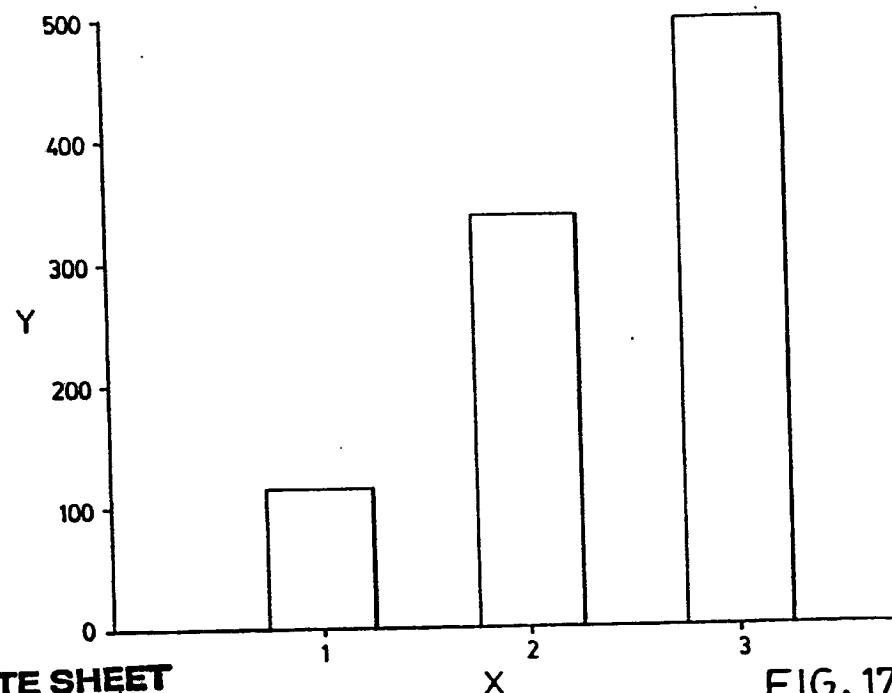

FIG. 17A

FIG. 17B

INTERNATIONAL SEARCH REPORT

International Application No   PCT/CA 92/00481

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N5/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	COMPTES RENDUS DES SEANCES DE L'ACADEMIE DES SCIENCES. SERIE III: SCIENCES DE LA VIE vol. 299, no. 6, 30 July 1984, MONTREUIL FR pages 143 - 146 F. CORMIER ET AL. 'DÉVELOPPEMENT DES PROGÉNITEURS ÉRYTHRO'DES PRÉCOCES (BFUe) DE LA MOELLE OSSEUSE DE SOURIS DANS UN MILIEU DE CULTURE DÉPOURVU DE SÉRUM. EFFET DE L'HÉMINE.' see the whole document --- -/--	1-20

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18 FEBRUARY 1993	0 9. 03. 93

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	RYCKEBOSCH A.O.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category°	Citation of Document, with indication, where appropriate, of the relevant passages	
A	CHEMICAL ABSTRACTS, vol. 109, no. 25, 19 December 1988, Columbus, Ohio, US; abstract no. 228285b, Y. SONODA ET AL. 'ERYTHROID BURST-PROMOTING ACTIVITY OF PURIFIED RECOMBINANT HUMAN GM-CSF AND INTERLEUKIN-3: STUDIES WITH ANTI-GM-CSF AND ANTI-IL-3 SERA AND STUDIES IN SERUM-FREE CULTURES.' page 632 ; cited in the application see abstract & BLOOD vol. 72, no. 4, 1988, pages 1381 - 1386	1-20
A	CHEMICAL ABSTRACTS, vol. 106, no. 3, 19 January 1987, Columbus, Ohio, US; abstract no. 13591a, G. KONWALINKA ET AL. 'HUMAN ERYTHROPOIESIS IN VITRO AND THE SOURCE OF BURST-PROMOTING ACTIVITY IN A SERUM-FREE SYSTEM.' page 150 ; cited in the application see abstract & EXP. HEMATOL. (N.Y.) vol. 14, no. 10, 1986, pages 899 - 903	1-20
P,X	BLOOD vol. 78, no. 11, 1 December 1991, NEW YORK, N.Y., US pages 2823 - 2833 P.N. CORREA ET AL. 'PRODUCTION OF ERYTHROPOIETIC BURSTS BY PROGENITOR CELLS FROM ADULT HUMAN PERIPHERAL BLOOD IN AN IMPROVED SERUM-FREE MEDIUM: ROLE OF INSULINLIKE GROWTH FACTOR 1.' see the whole document	1-20
P,X	INTERNATIONAL JOURNAL OF CELL CLONING vol. 10, no. 5, 1992, DAYTON, OH, US pages 286 - 291 P.N. CORREA ET AL. 'RETINYL ACETATE AND ALL-TRANS-RETINOIC ACID ENHANCE ERYTHROID COLONY FORMATION IN VITRO BY CIRCULATING HUMAN PROGENITORS IN AN IMPROVED SERUM-FREE MEDIUM.' see the whole document	1-20